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Na^+–H^+ exchange in luminal-membrane vesicles from rabbit proximal convoluted and straight tubules in response to metabolic acidosis

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Na^+–H^+-exchanger activity of pars convoluta and pars recta luminal-membrane vesicles prepared from the proximal tubule of acidotic and control rabbits were assayed by a rapid-filtration technique and an Acridine Orange method. Both experimental approaches revealed the existence of an antiporter, sensitive to metabolic acidosis, in pars convoluta membrane vesicles. Kinetic data, obtained with the pH-sensitive dye, showed that the K_m for Na^+ transport was unchanged by acidosis, whereas $V_{max.}$ for exchanger activity was increased, on an average, by 44%. The fluorescence method, in contrast with the rapid-filtration technique, was able to detect exchanger activity in pars recta membrane vesicles. The K_m value for the antiporter located in pars recta is comparable with that calculated for pars convoluta membrane vesicles. By contrast, the $V_{max.}$ of this exchanger is only about 25% of that found for pars convoluta. Furthermore, metabolic acidosis apparently does not increase Na^+–H^+-exchanger activity of pars recta luminal-membrane vesicles.

INTRODUCTION

In the proximal tubule the luminal Na^+–H^+ antiporter is the predominant system by which H^+ is secreted (Aronson, 1983). In accordance with expectations, Na^+–H^+-exchange activity was shown to be increased in metabolic acidosis. This has been revealed by using various techniques, among others by studies with luminal-membrane vesicles prepared from whole renal cortex taken from normal and acidotic rabbits (Tsai *et al.*, 1984), rats (Kinsella *et al.*, 1984*a*,*b*) or dogs (Cohn *et al.*, 1983). In contrast with the Na^+–H^+ antiporter, amiloride-insensitive Na^+ uptake and passive H^+ permeability were not altered by acidosis (Kinsella *et al.*, 1984*a*; Tsai *et al.*, 1984; Cohn *et al.*, 1983). With membrane vesicles prepared from whole renal cortex it is not possible to determine whether the antiporter is present in all segments of the proximal tubule and, therefore, whether the increase of Na^+–H^+-exchange activity takes place in all parts of that tubular structure.

Recently we have studied the activity of the Na^+–H^+ exchanger in luminal-membrane vesicles prepared from either pars convoluta ('outer cortex') or pars recta ('outer medulla') of the proximal tubule by a rapid-filtration technique (Kragh-Hansen *et al.*, 1985). The experimental findings showed that, in normal animals, the antiporter is predominantly operative in pars convoluta and probably of minor significance in pars recta. We have extended these studies as follows. Pars convoluta and pars recta luminal-membrane vesicles were prepared from both normal and acidotic rabbits and the Na^+–H^+-exchange activity of high concentrations of membrane vesicles was investigated not only by the rapid-filtration method but also by the Acridine Orange technique described by Warnock *et al.* (1982). By using these approaches we were able to detect antiporter activity in both segments of the proximal tubule. The affinity for Na^+ was the same whether the cation was taken up by pars convoluta or pars recta luminal-membrane vesicles or whether the membrane vesicles were prepared from normal or acidotic rabbits. However, acidosis increased the $V_{max.}$ for Na^+ uptake by pars convoluta luminal-membrane vesicles. By contrast, we were unable, by either technique, to detect any significant change in the $V_{max.}$ for the antiporter of pars recta membrane vesicles in acidosis.

EXPERIMENTAL

Generation of metabolic acidosis

New Zealand White rabbits (2.5–3.5 kg) were given drinking water containing 75 mM-NH_4Cl (Tsai *et al.*, 1984) *ad lib* and, after 2 days of starvation, a diet containing oatmeal, sucrose and NaCl as described by Poulsen & Praetorius (1954) for 3 days. Control animals were given drinking water containing 75 mM-$NaHCO_3$ and normal rabbit chow for all 5 days. The degree of acidosis introduced in the rabbits was determined by measuring plasma pH routinely and urine pH in all animals. Plasma pH and urine pH were 7.11 ± 0.04 ($n = 20$) and 5.43 ± 0.61 ($n = 79$) respectively in the rabbits given NH_4Cl and the diet, whereas the values were 7.42 ± 0.06 ($n = 20$) and 8.55 ± 0.20 ($n = 79$) respectively in the control animals.

Preparation of luminal-membrane vesicles

Immediately after removal of the kidneys from the rabbits they were perfused via the renal artery with 35 ml of homogenizing solution (310 mM-sorbitol/15 mM-Hepes/Tris, pH 6.0). We used five acidotic and five control animals for each set of experiments. After perfusion of the kidneys, tissue from outer cortex (pars convoluta of the proximal tubule) and from outer stripe

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of outer medulla (pars recta) was dissected as previously described (Kragh-Hansen *et al.*, 1984, 1985). The tissue fragments were homogenized, and four different membrane-vesicle preparations were made in parallel at 4 °C and pH 6.0 by using the Ca^{2+}-precipitation procedure previously described (Sheikh *et al.*, 1982). The membrane-vesicle suspensions were stored on ice and used within 6 h.

The protein concentrations were determined by the method of Lowry *et al.* (1951), as modified by Peterson (1977), with human serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as a standard. Measurements of marker-enzyme activities were performed routinely with results comparable with those previously published (Kragh-Hansen *et al.*, 1984, 1985). As judged by D-glucose-uptake studies no, or only a minor, cross-contamination of pars convoluta and pars recta membrane vesicles takes place (Kragh-Hansen *et al.*, 1984, 1985).

Transport studies

Time courses for uptake of 1 mM sodium by luminal-membrane vesicles from pars convoluta or pars recta from acidotic or control rabbits were determined by the rapid-filtration technique previously described (Kragh-Hansen *et al.*, 1985). For experimental details, see the legend to Fig. 1. $^{22}NaCl$ was obtained from New England Nuclear, Boston, MA, U.S.A., and amiloride was donated by Merck, Sharp and Dohme, Copenhagen, Denmark.

Initial uptake of various concentrations of Na^+ by the four different types of luminal-membrane vesicles was assayed by using an Acridine Orange method essentially identical with that described by Warnock *et al.* (1982). For a description of the method and experimental details, see the Results section and the legend to Fig. 3. The dye was purchased from George T. Gurr, London S.W.6, U.K.

Calculations

The Michaelis–Menten kinetics of the uptake of various of concentrations of Na^+ were analysed. Theoretical saturation curves were fitted to the experimental data by using a computer-analysed statistical iteration procedure (Jacobsen *et al.*, 1982). Statistical comparisons were made by Student's *t* test.

RESULTS

Transport measured by a rapid-filtration technique

Uptake of Na^+ by luminal-membrane vesicles prepared from outer cortex. The time courses for the uptake of 1 mM-Na^+, in the presence of an intravesicular > extravesicular H^+ gradient, by membrane vesicles derived from acidotic and control rabbits are shown in Fig. 1. It is seen that metabolic acidosis results in an increased initial uptake of Na^+. Furthermore, the magnitude of the transient overshoot is significantly higher ($P < 0.01$) in acidosis than in the controls. In acidosis the 90 s uptake values are about three times those measured at equilibrium, whereas the ratio is only about two for the controls. The lowest curve in Fig. 1 shows the effect of amiloride on the uptake of Na^+. The drug is a strong inhibitor of Na^+–H^+ exchanges (Kinsella & Aronson, 1980), and it can be seen from the Figure that amiloride totally eliminates the Na^+ overshoots and suppresses Na^+ uptake to about 28–34% of the original levels. This finding is in accordance with the existence of a Na^+–H^+ exchanger in pars convoluta.

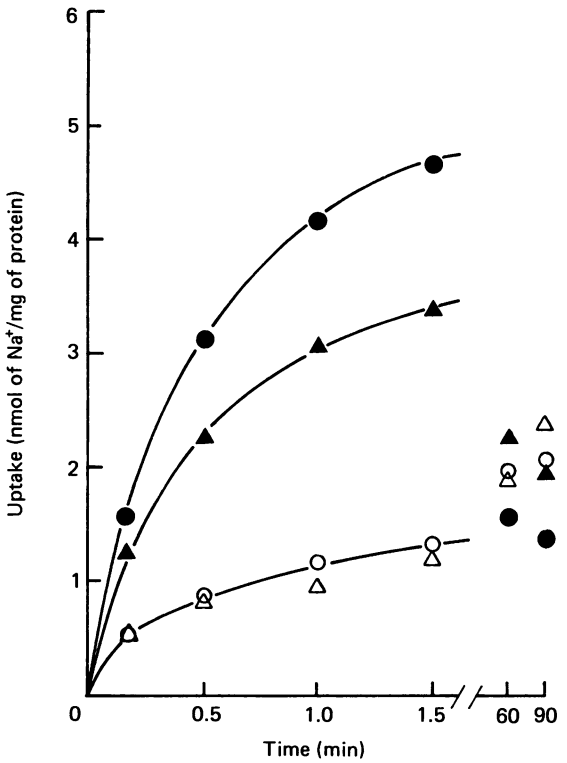

Fig. 1. Uptake of Na^+ by pars convoluta luminal-membrane vesicles

Transport was measured by a rapid-filtration technique. At zero time 100 μl of incubation medium was added to 20 μl of membrane-vesicle suspension. After various incubation periods at 20 °C, uptake of Na^+ was stopped by adding 1 ml of ice-cold incubation medium without radioactive Na^+. The incubation media, pH 7.2, contained 179 mM-mannitol, 18 mM-Mes, 47 mM-Tris, 79 mM-Hepes and 1 mM-NaCl (plus tracer amounts of $^{22}NaCl$). The membrane vesicles were preloaded for 1 h in 191 mM-mannitol/91 mM-Mes/29 mM-Tris/14 mM-Hepes, pH 5.9. Membrane vesicles prepared from acidotic (●, ○) or control animals (▲, △). The incubation media were with (○, △) or without (●, ▲) amiloride at a final concentration of 3.5×10^{-4} M. The initial protein concentrations were 16.7 ± 3.0 mg/ml (●, ○) or 15.8 ± 2.2 mg/ml (▲, △). The data are average values for four experiments with duplicate determinations.

Uptake of Na^+ by luminal-membrane vesicles prepared from outer medulla. It is evident from a comparison of Fig. 1 with Fig. 2 that great differences exist between Na^+ uptake by pars convoluta and pars recta membrane vesicles in the presence of intravesicular > extravesicular H^+ gradients. First, Na^+ uptake by pars recta membrane vesicles from acidotic and normal animals do not differ significantly ($0.30 < P < 0.40$). Second, neither uptake of the ion by membrane vesicles prepared from acidotic rabbits nor uptake by membrane vesicles derived from control animals exhibit transient overshoots. The 90 s uptake values for both curves are only about half of those measured after 60–90 min of incubation.

These findings indicate that the Na^+–H^+ exchanger is either lacking, or of minor significance, in the proximal

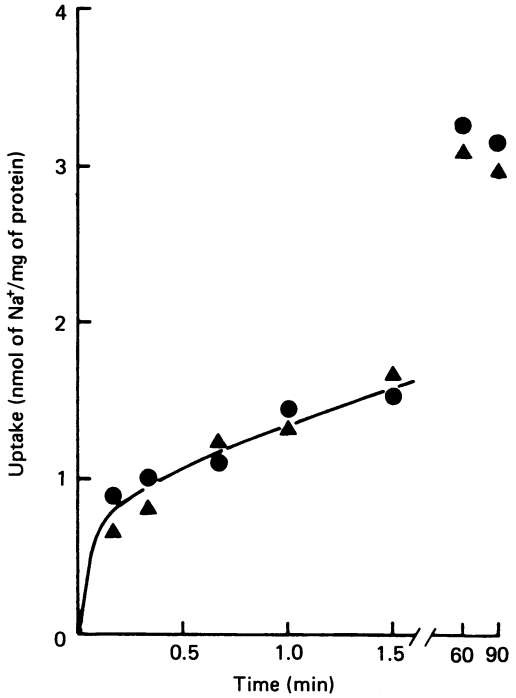

Fig. 2. Uptake of Na^+ by pars recta luminal-membrane vesicles

Transport was measured by a rapid-filtration technique, and the experimental protocol was identical with that described in the legend to Fig. 1. The initial protein concentrations were 16.0 ± 2.5 mg/ml and 17.1 ± 3.1 mg/ml for acidotic (●) and control (▲) membrane vesicles respectively. The data are average values for four experiments with duplicate determinations.

straight tubule. Another possible explanation for the lacking/reduced Na^+–H^+-antiporter activity could be that the rapid-filtration technique, in our hands, is not suitable for the detection of such an activity in pars recta luminal-membrane vesicles. Therefore we re-examine the segmental activity of the Na^+–H^+ exchanger by using the Acridine Orange method of Warnock *et al.* (1982).

Transport measured by the Acridine Orange uptake method

The Acridine Orange technique was chosen as the alternative method because it can provide the time resolution needed for the measurement of initial transport rates (Warnock *et al.*, 1982). The dye is a weak base with characteristic excitation (493 nm) and emission maxima (530 nm). Both the protonated and the non-protonated form can fluoresce. However, it is assumed that only the non-protonated form of the dye can pass through the membranes. This will result in an accumulation of protonated Acridine Orange in acidic compartments. Moreover, it is supposed that intravesicular dye is quenched to such an extent that it does not contribute to the measured fluorescence (Warnock *et al.*, 1982). Thus, if the intravesicular medium is more acidic than the outer medium, Acridine Orange will accumulate in the intravesicular space and the dye fluorescence will be quenched (and vice versa).

In Fig. 3(*a*) is shown a typical fluorescence recording. First, Acridine Orange and external medium, pH 7.2, were mixed. Later, a portion of luminal-membrane vesicles was added. Since the membrane vesicles had been preloaded at pH 5.9, a quenching of the dye fluorescence was observed. After a new equilibrium was established, an appropriate amount of a sodium D-gluconate stock solution was added in order to obtain an initial external Na^+ concentration of 90 mM. It is seen that the presence of Na^+ results in fluorescence recovery. This event reflects the Na^+–H^+-exchange activity of the membrane vesicles, and determinations of initial rates after addition of different amounts of Na^+, as illustrated by the tangent in the Figure, can be used to obtain kinetic parameters for the exchanger (Warnock *et al.*, 1982).

The initial rates of fluorescence recovery (v) determined for different Na^+-concentrations (s) are given in Fig. 3(*b*) as a v-versus-v/s plot. As seen in the Figure, the data followed a regression line, indicating that the results can be described by Michaelis–Menten kinetics.

Initial-rate determinations were also made for a constant Na^+ concentration but at different protein concentrations. Since a linear relationship between these two parameters exists (Fig. 3*c*), the method is independent of the protein concentration in the range used in the present study.

In conclusion, the Acridine Orange method seems well suited for a kinetic study of the Na^+–H^+ exchanger of not only luminal-membrane vesicles prepared from whole renal cortex but also of membrane vesicles derived from the convoluted or the straight part of the proximal tubule.

Na^+–H^+-exchanger activity by luminal-membrane vesicles prepared from outer cortex. The initial rates (2 s) of Acridine Orange fluorescence changes after addition of various amounts of Na^+ to luminal-membrane vesicles prepared from pars convoluta were determined. The results of a typical experiment using membrane vesicles from both acidotic and control animals and an intravesicular > extravesicular H^+ gradient are given in Fig. 4. It is seen that the uptake values calculated for the acidotic membrane vesicles are greater than those of the controls. The two sets of data were analysed by using a computer program in order to calculate K_m and $V_{max.}$ for Na^+ transport. The average values ($\pm$S.E.M.) for seven different sets of preparations were, in the case of acidosis, $K_m = 27 \pm 3$ mM and $V_{max.} = 5.0 \pm 0.2$ fluorescence unit$\cdot s^{-1} \cdot$mg of protein^{-1} and, for the controls, $K_m = 29 \pm 3$ mM and $V_{max.} = 3.6 \pm 0.2$ fluorescence units$\cdot s^{-1} \cdot$mg of protein^{-1}. The K_m values are not significantly different. By contrast, acidosis results in a significant increase of $V_{max.}$ ($P < 0.001$).

The effect of amiloride is illustrated by the open symbols in Fig. 4. It is seen that the drug suppresses the initial rates for the two types of membrane vesicles to the same low level.

The results shown in Fig. 4 strongly indicate the presence of a Na^+–H^+ exchanger, sensitive to metabolic acidosis, in pars convoluta membrane vesicles. The data are in accordance with the results obtained with the rapid-filtration technique (Fig. 1), since the latter exhibited an amiloride-sensitive overshoot, the magnitude of which was increased in acidosis.

Na^+–H^+-exchanger activity by luminal-membrane vesicles prepared from outer medulla. Fig. 5 shows the initial fluorescence rates accompanying uptake of various Na^+ concentrations by acidotic and control luminal-membrane vesicles representing pars recta. In

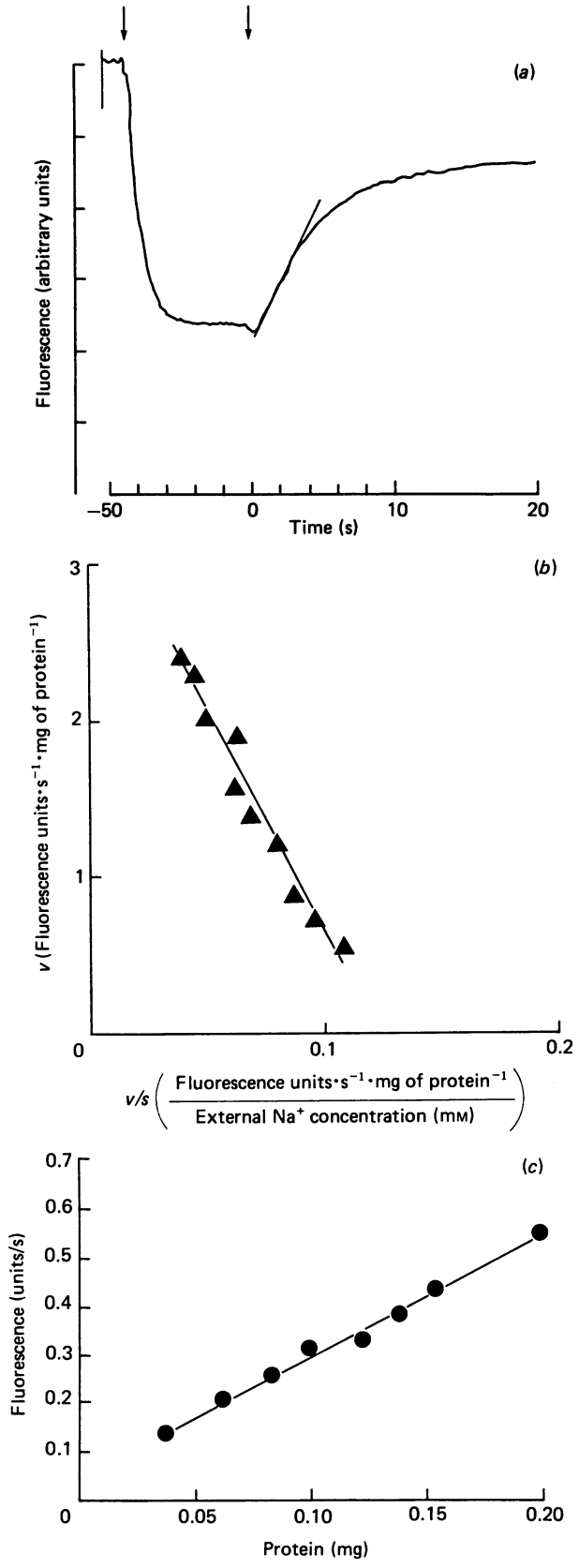

Fig. 3. Na^+–H^+ exchange activity as assayed by Acridine Orange fluorescence

(*a*) A 2.5 ml portion of Acridine Orange (final concn. 6 μM) dissolved in 179 mM-mannitol/18 mM-Mes/47 mM-Tris/79 mM-Hepes, pH 7.2, was pipetted into a cuvette placed in an Aminco–Bowman spectrophotofluorimeter equipped with a recorder. The solution was excited at 493 nm and the dye emission was registered at 530 nm. Later, at the first break of the recording, were added 10 μl of a pars convoluta luminal-membrane-vesicle suspension prepared from controls and preloaded for 1.5 h at 0 °C in a medium containing 191 mM-mannitol, 91 mM-Mes, 29 mM-Tris and 14 mM-Hepes, pH 5.9. At the second break of the fluorescent trace, 100 μl of a sodium D-gluconate stock solution was added, resulting, in this example, in a final concentration of 90 mM. The initial rate of fluorescence recovery was calculated by using the slope of the tangent drawn through the recording obtained within the first 2 s after addition of Na^+. The content of the cuvette was stirred throughout the experiment and the temperature was 20 °C. The initial protein concentration was 9 mg/ml. (*b*) Initial rates of the Na^+–H^+ exchanger calculated after addition of various amounts of Na^+ plotted against initial rates divided by external Na^+ concentrations. (*c*) Initial rates of the Na^+–H^+ exchanger calculated after addition of a constant amount of Na^+ (final concn. 90 mM) plotted as a function of protein concentration. The results given in panels (*b*) and (*c*) are those of a typical experiment.

contrast with the results calculated when similar experiments were carried out with pars convoluta membrane vesicles (Fig. 4), the present data can be described by a common saturation curve. The similarity of the results for acidotic and control membrane vesicles is strengthened by a calculation of K_m and $V_{max.}$ for Na^+ uptake. The K_m values for acidotic and control membrane vesicles ($n = 7$) were 32 ± 5 mM and 32 ± 4 mM respectively, and $V_{max.}$ values were 0.9 ± 0.04 fluorescence units·s^{-1}·mg of $protein^{-1}$ and 0.8 ± 0.03 fluorescence units·s^{-1}·mg of $protein^{-1}$. The lack of an

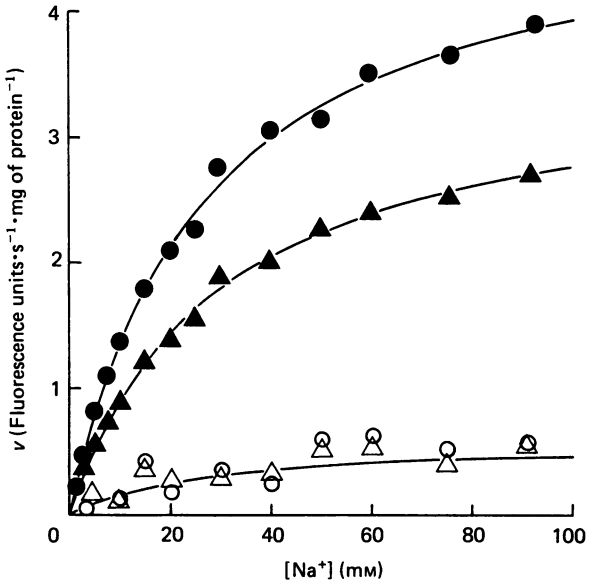

Fig. 4. Na^+–H^+-exchange activity of pars convoluta membrane vesicles as assayed by Acridine Orange

Initial rates of fluorescence recovery after addition of various amount of Na^+ to luminal-membrane vesicles prepared from acidotic (●) or control (▲) animals are shown. The experimental protocol was identical with that described in the legend to Fig. 3, except for the experiments carried out in the presence of amiloride (○, △) at a final concentration of 3.5×10^{-4} M. The results are from a typical experiment.

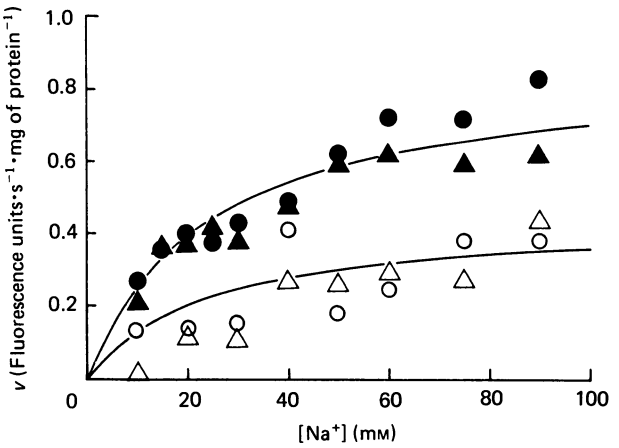

Fig. 5. Na^+–H^+-exchange activity of pars recta membrane vesicles as assayed by Acridine Orange

Initial rates of fluorescence recovery after addition of various amounts of Na^+ to luminal-membrane vesicles prepared from acidotic or control animals are shown. The experimental protocol was identical with that described in the legends to Figs. 3 and 4. The symbols used for control and acidotic animals are the same as those shown in Fig. 4.

increment of initial rate in acidosis is in accordance with the results of the rapid-filtration technique (Fig. 2), which showed comparable uptakes of Na^+ by membrane vesicles from acidotic and control animals.

In order to examine whether the fluorescence data of Fig. 5 reflects Na^+–H^+-exchanger activity, the experiments were also performed in the presence of amiloride. As seen by the open symbols in the Figure, the drug actually decreased Na^+ uptake to about 50% of the level measured in the absence of amiloride. That observation strongly suggests the existence of a Na^+–H^+ exchanger in pars recta membrane vesicles. These observations are not in accordance with the results of the rapid-filtration studies, since no overshoots were observed in these experiments (Fig. 2). An immediate explanation for the divergence between the two methods at this point could be that the dye method is more sensitive than the rapid-filtration technique.

DISCUSSION

The present study clearly demonstrated that Na^+–H^+-exchange activity increased in response to metabolic acidosis in luminal-membrane vesicle preparations derived from pars convoluta of rabbit kidney proximal tubule. Furthermore, this increased influx of Na^+ under pH-gradient conditions ($pH_{in} < pH_{out}$) is specifically inhibited by amiloride. The affinity for Na^+–H^+ exchange is unchanged in vesicles obtained from acidotic rabbits. These results are in agreement with previous studies performed with luminal-membrane vesicles prepared from whole renal cortex of rats, dogs and rabbits (Cohn *et al.*, 1983; Kinsella *et al.*, 1984*a*,*b*; Tsai *et al.*, 1984). Kinsella *et al.* (1984*a*) have extensively studied the various kinetic parameters of Na^+–H^+ antiporter in vesicles from acidotic animals. On the basis of kinetic analysis of the experimental data, they concluded that metabolic acidosis leads to either an increase in the number of functioning exchangers or an increase in the turnover of a rate-limiting step in the exchange. Subsequent studies from their laboratory (Kinsella *et al.*, 1984*b*) further showed that increased amiloride-sensitive Na^+–H^+-exchange activity in metabolic acidosis required an intact adrenal gland or glucocorticoid supplements, namely dexamethasone. These findings are consistent with glucocorticoids having an important regulatory role in the kidney by orchestrating the proximal-tubular adaptation to metabolic acidosis. In this connection it is important to note that Mishina *et al.* (1981) have found that the rat renal proximal tubule was 3–6-fold enriched in glucocorticoid receptors as compared with the distal-tubular content. However, in that study the amount of glucocorticoid receptors in pars convoluta and pars recta of rat renal proximal tubule was not examined. From these studies one may conclude that an increased rate of synthesis of Na^+–H^+ exchanger, in response to metabolic acidosis, is to account for the increase in $V_{max.}$.

In a more recent study, Vigne *et al.* (1985) have examined the characteristics of the Na^+–H^+ antiporter in luminal-membrane vesicles from normal and hypertrophied rabbit kidneys. They found that compensatory adaptation of the kidney to a chronic decrease in renal mass was accompanied by a 1.7-fold increase in the activity of the Na^+–H^+-exchange system. Furthermore, they reported that the properties of interaction of intravesicular and extravesicular pH with the Na^+–H^+ antiporter of normal and hypertrophied kidneys were identical, indicating that the properties of the intravesicular H^+ modifier site, the existence of which was originally demonstrated by Aronson *et al.* (1982), was unchanged in membrane vesicles from whole renal cortex of hypertrophied kidney. They further studied the binding characteristics of a radioactive amiloride derivative, [^{3}H]ethylpropylamiloride, to the Na^+–H^+-exchange system from normal and hypertrophied rabbit kidneys. However, the results obtained by titration of [^{3}H]ethylpropylamiloride-binding sites in normal and hypertrophied kidneys suggested that the increased activity of the Na^+–H^+ exchange system is not accompanied by an increased concentration of exchanger. They therefore concluded that existing carriers have a more rapid turnover in hypertrophied kidneys. However, they pointed out that this conclusion presupposes that all Na^+–H^+-exchange systems that can be titrated with [^{3}H]ethylpropylamiloride correspond to functional antiporters in luminal membranes.

An alternative explanation for the increased $V_{max.}$ values in vesicles from acidotic animals is the probable formation of endocytotic vesicles during metabolic acidosis. It has been demonstrated that renal proximal-tubular cells secrete and ingest macromolecules by the process of exocytosis and endocytosis. There is increasing evidence that these processes are used to recycle the plasma membrane. Recent studies have identified rapid fusion as a mechanism for regulation of the number of surface membrane proteins. If the same phenomenon exists in the renal proximal tubule undergoing hypertrophy, then it has to be assumed that endocytic vesicles carrying 'silent' Na^+–H^+ antiporters were co-purified with the luminal-membrane vesicles preparation from hypertrophied kidney, as has been discussed by Vigne *et al.* (1985). It seems preferable at present to avoid further discussion on the mechanism of adaptation of Na^+–H^+ antiporter in response to abovementioned external stimuli.

Another interesting feature of the present study is the demonstration of Na^+–H^+-exchanger activity in luminal-membrane vesicles isolated from pars recta of rabbit proximal tubule. In a previous report we were unable to demonstrate the presence of Na^+–H^+ antiporters in these vesicle preparations (Kragh-Hansen *et al.*, 1985). As mentioned in the Results section, the possible explanation may be the fact that the rapid-filtration technique is not suitable for this purpose. By using an Acridine Orange method (Warnock *et al.*, 1982) we found that Na^+–H^+-exchange activity is much less (about 25% of that of pars convoluta membrane vesicles) in luminal-membrane vesicles from pars recta. Our present observations are in accordance with the findings on cellularly intact systems by McKinney & Burg (1977), who have studied in microperfusion experiments the absorption of HCO_3^- by straight proximal tubules (pars recta) under various experimental conditions. They found that the Na^+-dependent rate of absorption of HCO_3^- in the proximal straight tubule was approximately 25% of the rate in the convoluted tubule. Iino & Burg (1981) using microperfusion experiments, examined both the Na^+-dependent and Na^+-independent absorption of HCO_3^- by straight proximal tubule dissected from acidotic rabbit renal cortex. They found that, in straight proximal tubule, neither the Na^+-dependent nor the Na^+-independent component of bicarbonate absorption was significantly affected by the acid–base status of the animal from which the tubules were obtained. These findings are in good agreement with our observations that the Na^+–H^+ antiporter activity in vesicles from pars recta is not significantly changed in response to metabolic acidosis.

In conclusion, the results presented here showed that the luminal-membrane vesicles both from pars convoluta and pars recta, of rabbit proximal tubule contain Na^+–H^+-exchange system(s) with similar affinities (K_m values), but with different $V_{max.}$ values. Furthermore, an increase in $V_{max.}$ of the Na^+–H^+ exchanger in response to metabolic acidosis is only observed in vesicles from pars convoluta. Our experimental data cannot differentiate whether the increase in $V_{max.}$ is a consequence of an increased number of functioning carriers or due to an increase in the turnover rate of the limiting step in the exchange.

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